ONLINE DATA SUPPLEMENT

PROXIMAL TUBULE ANGIOTENSIN AT_2 RECEPTORS MEDIATE AN ANTI-INFLAMMATORY RESPONSE VIA INTERLEUKIN-10: ROLE IN RENOPROTECTION IN OBESE RATS

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EXTENDED METHODS

Chemicals and Reagents

Human proximal tubule epithelial cells (HK-2) were purchased from ATCC (Manassas, VA). Keratinocyte serum free medium (K-SFM), fetal bovine serum (FBS), penicillin/streptomycin, epidermal growth factor (EGF) and bovine pituitary extract (BPE) were purchased from Life Technologies (Carlsbad, CA). C21 was custom synthesized according to a previously published scheme (Wan et al., 2004). Polyclonal AT_1R antibody was custom raised by Biomolecular Integrations (Little Rock, AR). Polyclonal AT_2R antibody was custom raised by EZ Biolab (Carmel, IN). Anti-rat CD68 antibody, L-NAME, TGF-β and IL-10 antibodies, neutralizing IL-10 antibody, total nitrates and cytokine ELISA kits were purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

HK-2 cells were cultured at 37ºC, 5% CO_2 in K-SFM supplemented with 10% FBS, 1% penicillin/streptomycin, 5ng/ml EGF and 50μg/ml BPE. All experiments were performed on cells between passages 5-12 when the cells were 70-80% confluent. All experiments were performed in triplicate.

In vitro Protocols

HK-2 cells were seeded in 6-well plates and when 70-80% confluent, the media was replaced with in K-SFM without any supplememts . These cells were treated for 24 hours with LPS (10 ug/ml), C21 (1 umol/L or a dose range 0.1-10 umol/L) or both agents simultaneously for 24 hours. At the end of treatment, media was collected, filtered through 0.2 um filter and analyzed for total nitrates or cytokines by kit-based ELISA (R&D Systems). Total nitrates in the supernates were determined using a kit-based EIA (R&D Systems). To determine whether the effect of C21 on cytokines was indeed mediated by the AT_2R, cells were treated with AT_2R antagonist PD123319 (10 umol/L) 15 min before the addition of C21. For neutralizing the effect of IL-10, a specific IL-10 antibody (0.25, 0.5, 1.0 and 2.5 ug/ml) was added to the medium 15 min before the addition of LPS and/or C21. For inhibition of nitric oxide synthase, non-specific NOS inhibitor L-NAME (1 mmol/L) was added to the media 15 min before the addition of LPS and/or C21.

Animal Model

Male lean and obese Zucker rats (5 weeks of age) were purchased from Harlan Laboratories (Madison, WI). The animals were housed in the University of Houston animal care facility and had free access to standard rat chow and tap water. The Institutional Animal Use and Care Committee approved animal experimental protocols. The lean and obese rats (n=5-7) were divided into vehicle, AT_2R antagonist (PD123319), AT_2R agonist (C21), and AT_2R agonist-antagonist (PD123319+C21) treated groups. Vehicle (saline) and C21 (300 μg/kg/day) were injected daily i.p and PD123319 (50 μg/kg/min) was continuously infused for 2 weeks via subcutaneously implanted osmotic pumps (Alzet, Palo Alto, CA).

Immunoblotting for AT_1R and AT_2R Expression

Treated HK-2 cells were washed with cold phosphate buffered saline and lysed in 0.3 ml of lysis buffer containing 0.5 M Tris base (pH 6.8), 1% SDS, 1 mM EDTA, 1 mM PMSF, and protease inhibitor (aprotinin, calpain inhibitors, leupeptin, pepstatin, and trypsin inhibitor). The cell lysates were used for protein estimation by kit based BCA method. For determining AT_1R abundance 10 ug and 50 ug of protein from HK-2 cell lysate were subjected to SDS-PAGE and transferred onto immobilon P (blot). To determine the change in AT_2R expression with LPS, 50 μg of protein was loaded and subjected to SDS-PAGE.

For the determination of AT_1R abundance in renal cortex homogenates from lean and obese Zucker rats, increasing amounts of protein (5, 15, 30, 50 μg) from the sample were. Similarly, for AT_2R abundance increasing amounts of protein (10, 25, 50, 70 μg) of protein were loaded and subjected to western blotting for the protein of interest using respective protein specific polyclonal antibodies. Polyclonal IgG-linked with horseradish peroxidase and the ECL system were used to detect the signal, which were analyzed by FluorChem 8800 (Alpha Innotech Imaging System, San Leandro, CA). β-actin was used as a loading control.

Blood Pressure Measurement

Blood pressure was measured in conscious, restrained rats using CODA System tail-cuff plethysmography (Kent Scientific). Rats were trained for 3 days prior to taking final BP measurements. This system has been clinically validated and the results have been shown to provide 99% correlation with telemetry measurements of BP.

Renal Histology and Morphometry

In order to assess the pathological changes that occur in the kidneys of the lean and obese Zucker rats, mesangial matrix expansion, focal segmental glomerulosclerosis development, tubulo-interstitial fibrosis and macrophage infiltration was assessed. After sacrificing the animals under anesthesia with inactin (150 mg/kg i.p), the kidneys were flushed with cold phosphate buffered saline to remove blood and then be fixed in formalin for 24 hours at 4°C. The formalin-fixed tissue was embedded in paraffin and 4 μm sections were prepared. The slides were further stained with Periodic Acid Schiff reagents.

Periodic Acid Schiff (PAS) Staining: To evaluate the gross tubular and glomerular structural changes, PAS staining was performed on the sections using a kit-based technique (Dako North America, Inc.) according to the manufacturer's instructions. All tissue samples were evaluated independently by two investigators in a blinded fashion by light microscopy (x400). For each animal, 30 glomeruli and 100 tubules from each of the 3 consecutive sections were assessed. A semi-quantitative scoring method described by Raij et al. (1984) was used to evaluate the degree of damage to the glomeruli or tubules. This was graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhesion involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; and 4, sclerosis >75% of the glomerulus. The glomerulosclerosis index was calculated by averaging the grades assigned to all glomeruli fields using the formula: **Sclerosis Index = (N1 x 1 + N2 x 2 + N3 x 3 + N4 x 4)/ n;** where N1-N4 are number of glomeruli with the respective score, n is the total number of glomeruli.

Immunostaining for CD68: CD68 is a cytosolic antigen protein specific for monocytes and macrophages. Immunostaining was carried out according to standard procedures using anti-CD68 (R&D Systems), and biotinylated secondary antibodies (Vector Laboratories) and revealed with avidin-peroxidase (Vectastain Elite; Vector Laboratories Inc.). Slides were mounted with VectaMount (Vector Laboratories) and photographed under a Nikon Eclipse TS100 microscope (Nikon Instruments Inc.) using an Infinity 1 digital camera (Media Cybernetics Inc.). The number of CD-68 positive cells per glomerulus was reported. An average of 30 glomeruli per section and 3-consecutive sections per kidney were analyzed.

Statistical Analysis

Data are presented as means ±SE. To analyze variations between groups, Student's *t*-test was used. One-way ANOVA with post-hoc (Newman-Keuls) tests was used to compare variations within groups. A value of $p<0.05$ was considered statistically significant, with n=5–7 experiments or rats per group.

SUPPLEMENTAL RESULTS

AT_1R and AT_2R Expression in PTECs: A single band (~41 kDa) for AT_1R was detected even at 10 µg of protein loading (Fig S1). Western blotting showed the presence of the AT_2R as two distinct bands (~45 and ~40 kDa) in HK-2 cells at 50 µg of protein loading (Fig. S2). No band was detected at 10 µg protein (data not shown). The two bands are likely due to varying degrees of glycosylation as has been previously reported (Hakam and Hussain, 2005, Kornfeld and Kornfeld, 1985). LPS treatment significantly downregulated AT_2R expression in activated PTECs (Fig S2).

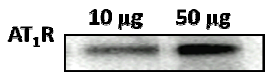

Fig. S1: AT_1R Expression in HK-2 cells. Representative western blot for AT_1R (~41 kDa) showing a detectable band with both 10 µg and 50 µg of loaded protein.

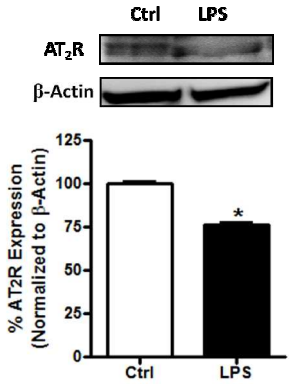

Fig. S2: AT_2R Expression in HK-2 proximal tubule epithelial cells. Representative western blots for AT_2R protein (approx. 40 and 45 kDa) top panel and for β–actin in the lower panel. Data are mean ± SEM (n = 3). * indicates $p<0.001$ vs control untreated HK-2 cells.

Effect of AT_2R agonist C21 on cytokine production by TNF-α activated PTECs: Similar to LPS, TNF-α (10 ng/ml) was able to significantly increase the production of pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 (Fig. S3).

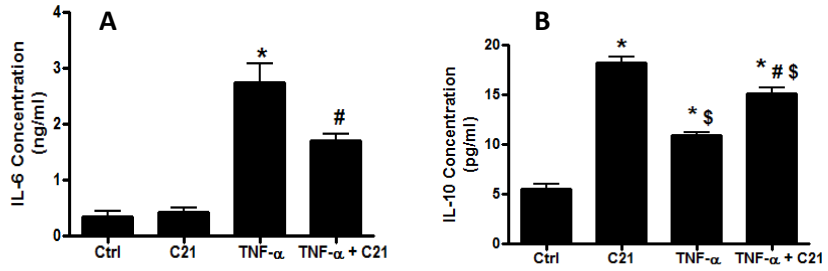

Fig. S3: (A) Interleukin-6 (IL-6) and (B) interleukin-10 (IL-10) concentration in the media collected from HK-2 cells stimulated by TNF-α (10 ng/ml) and/or C21 (1 umol/L). Data are mean ± SEM (n = 3). * indicates $p<0.05$ vs control untreated, # indicates $p<0.05$ vs TNF-α treated and $ indicates $p<0.05$ vs C21 treated HK-2 cells.

Dose response effect of AT_2R agonist on IL-10 production by LPS-activated PTECs: Increasing doses of C21 (0.1-10 µmol/L) led to a corresponding increase in IL-10 levels in the media even in the absence of LPS activation. This effect was blocked by concurrent addition of AT_2R antagonist PD123319, confirming that the NO production was indeed AT_2R mediated (Fig. S4).

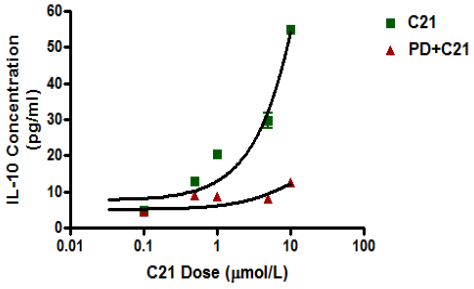

Fig. S4: Dose dependent increase in interleukin-10 (IL-10) production by HK-2 proximal tubule epithelial cells following treatment with AT_2R agonist (C21; 0.1-10 µmol/L) for 24 hours. AT_2R antagonist PD123319 (PD, 10 µmol/L) was able to block the C21-mediated increase in IL-10 concentration. Cytokine concentrations in media were measured by ELISA. Data are represented as mean ± SEM (n=5).

Effect of AT_2R agonist C21 on nitric oxide production by PTECs: In this set of experiments, HK-2 cells were treated with increasing doses of C21 and the production of nitrates/nitrites in the medium was determined after 24 hours, as an indicator of NO production. C21 treatment dose (0.1-10µmol/L) dependently increased the level of nitrates/nitrites and this increase was blocked by concurrent addition of AT_2R antagonist PD123319, confirming that the NO production was indeed AT_2R mediated (Fig. S5).

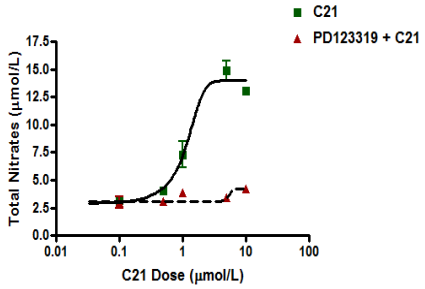

Fig. S5: Dose dependent increase in total nitrates in the media collected from HK-2 proximal tubule epithelial cells

following treatment with AT_2R agonist (C21; 0.1-10 μmol/L) for 24 hours. AT_2R antagonist PD123319 (PD, 10 μmol/L) was able to block the C21-mediated increase in total nitrate production. Data are represented as mean ± SEM (n=5).

AT_1 and AT_2 receptor abundance in lean and obese Zucker rats: Increasing amounts of protein were loaded for detection of AT_1R (5, 15, 30, 50 μg) and AT_2R (10, 25, 50, 70 μg) in lean and obese rats. In LZR and OZR a band for AT_1R (~41 kDa) was detected even at 5 μg. In OZR, a pair of faint bands (~44 and 39 kDa) was detected at 10 μg of loaded sample, while a band of similar intensity could be detected in the lean rat only at 50 μg of loaded sample. Taken together, these observations suggest that in both LZR and OZR, AT_1R is more abundant than AT_2R and that AT_2R is up-regulated in OZR compared to LZR (Fig. S6).

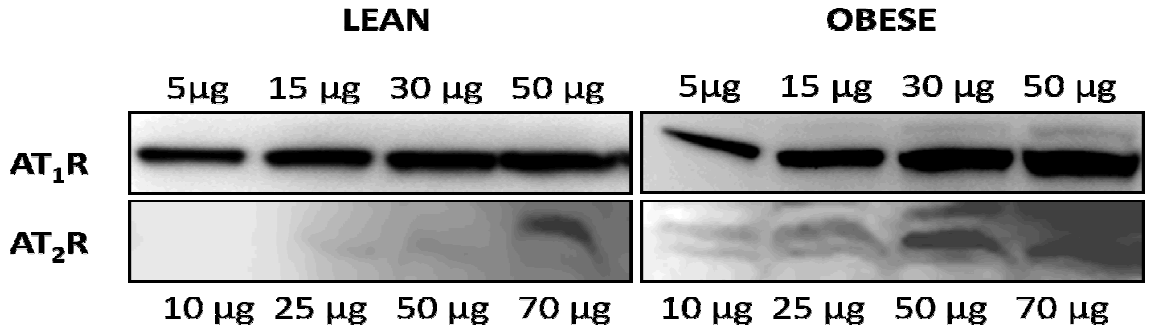

Fig. S6: Representative blots depicting the relative abundances of AT_1R and AT_2R in lean and obese Zucker rats.